Cultured Neuron Probe

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This QPR is being sent to you before it has been reviewed by the staff of the Neural Prosthesis Program.

General Introduction

Our aim is to develop a cultured neuron probe. This consists of a silicon structure into which individual dissociated neurons can be placed, and which can be inserted into an intact nervous system. Furthermore, within the structure each neuron is in close proximity to an electrode, by means of which it can be stimulated, or its activity can be recorded, through electrical leads which connect to external electronics. It is hoped that neurons in the probe will synaptically integrate with the host nervous system, to provide a highly specific, relatively non-invasive, two-way communication channel. If this occurs, the methodology has important possibilities for neural prostheses. The goal of this project is to perform initial experiments to establish the feasibility of communication by means of a neuron probe. The tissue we have chosen in which to initially implant the probe is the rat hippocampus. If initial studies are successful, probes will be designed and implanted for communication with sensorimotor cortex.

The neuron probe we plan to fabricate will be made of micromachined silicon and will have sixteen electrodes: one conventional electrode to detect activity during placement of the probe, and fifteen within wells into which neurons will be placed. Its configuration will be similar to that of passive multielectrode probes which have been developed. It will be implanted when the cultured neurons are very young, and after a time of weeks it is hoped that they will have survived and made two-way synaptic connections. By stimulation and recording in the host and probe, we will test for the existence of such connections. An essential feature of the experiments is that the viability of the implanted neurons will be independently determined over time by stimulating them and recording their resulting action potentials.

Summary

During this quarter the *in vitro* efforts were directed toward doing electrophysiology experiments with neurochips. The initial plan was to use symnpathetic neurons from the rat superior cervical ganglion (SCGs) because they are robust and easy to culture. Preliminary experiments were done in which it was shown that these neurons could easily be triggered by antidromic signals generated by stimulation of an axon. This is an ideal way to cleanly generate action potentials in a neurochip neuron in order to see the recording behavior. However, it was found that these SCG neurons were adept at escaping from the new canopy wells by growing processes out the top hole and climbing up and out. This took place on such a short time scale that physiology experiments could not reliably be done. Therefore, efforts have now been turned to using hippocampal neurons for the inititial tests, and a culture system for pure neuron cultures growing on neurochips has been developed. Initial physiology experiments with these cells will begin next quarter.

While culturing neurons on neurochips it was found that the lead connections from the chip to the support printed circuit were not reliable, and that the failure rate after more than a few days in the incubator atmosphere was prohibitively high. A new mounting and lead connection scheme has developed, using only ultrasonic bonding of gold wire to gold pads, which appears to be stable indefinitely.

Fabrication during the quarter has been successful in producing excellent dummy probes in quanitity, and also excellent neurochips in sufficient numbers for experiments during the coming quarter. A mystery persists about failure of the final EDP etch step in some neurochip wafers. When this has been understood, it is expected that a large number of good neurochips will also be in hand.

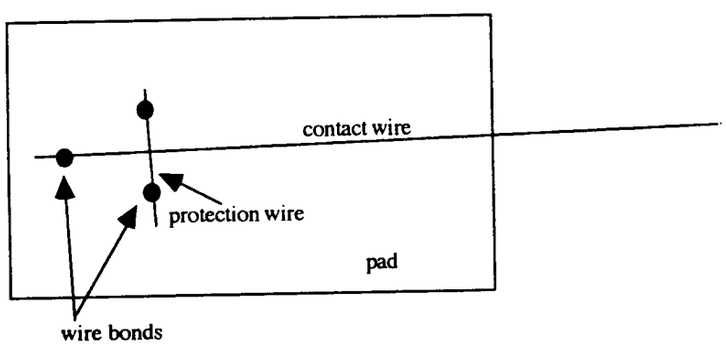
In vivo experiments have emphasized the outgrowth of septal cholinergic neurons, and observation of these cells after long-term experiments. It has been seen that some cells do sruvive for periods of months, but that outgrowth is minimal. However, these experiments were done using old dummy probes with wells which had an overhang, and this has been found to inhibit outgrowth.

Neurochips

Mounting

A necessity for a successful neurochip/neuroprobe assembly is the ability to maintain electrical contact from the electrode to the outside electronics. Our original plan was to use gold or silver epoxy to attach wires to the chip bonding pads, and then to connect these leads to a printed circuit chip carrier. Silicone elastomer used to seal the assembly, was found to be inadequate. In the continuous presence of water and water vapor in an incubator, the vapor penetrates silicones. Conducting epoxies then tend to soften and disintegrate and non-noble metals corrode, causing open circuits and loss of electrical continuity to the electrode. Gold epoxy-to-base metal contacts protected by Silastic would begin to fail after 3-7 days in the incubator, and after 2-4 weeks in the incubator, essentially all contacts would have failed.

Partially based on consultation with Dave Edell at MIT, we have designed a system using gold wires ultrasonically bonded between a chip carrier and the chip. Our carriers are standard 24-pin dual in-line discrete component carriers, with 0.6" spacing between the rows of pins. They are modified by filing the component-holding contacts flat and then gold plating them. The neurochip fits nicely at the center of this carrier. It is necessary to first wire-bond 16 leads to the bottom of the chip, leaving the ends dangling, and then to turn over the chip, attach it to the carrier with silastic, and bond the leads to the filed and gold plated carrier contacts. This technique leaves the chip wire bond vulnerable to breakage during assembly due to movement of the free wire, so this delicate bond is protected by a second, short wire-bonded wire close to the contact wire's bond. Further protection is provided by potting the entire back side of the chip with Silastic before bonding to the carrier. The sketch below indicates this schematically:



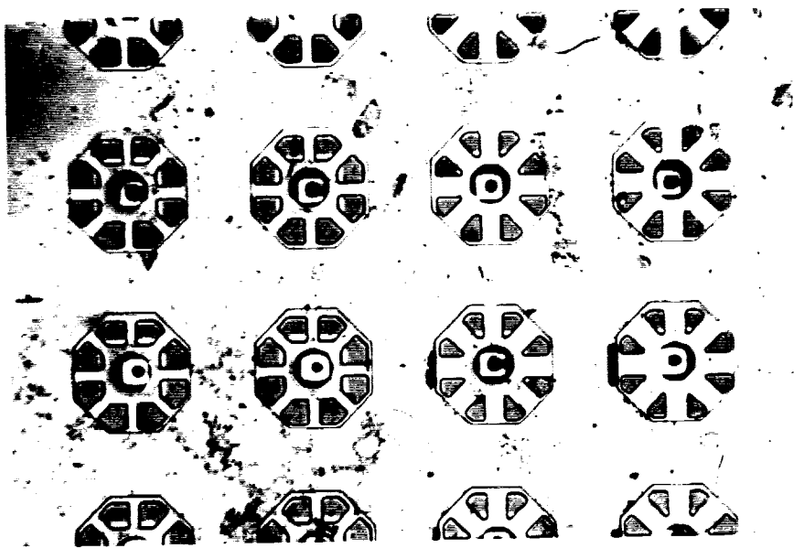
Using this mounting procedure, we have reduced the failure rate of contacts in the incubator from approximately one per chip per day to less than one per chip per month. We have in fact seen no contact failures on wire-bonded chips, even on chips in the incubator for up to 6 weeks.

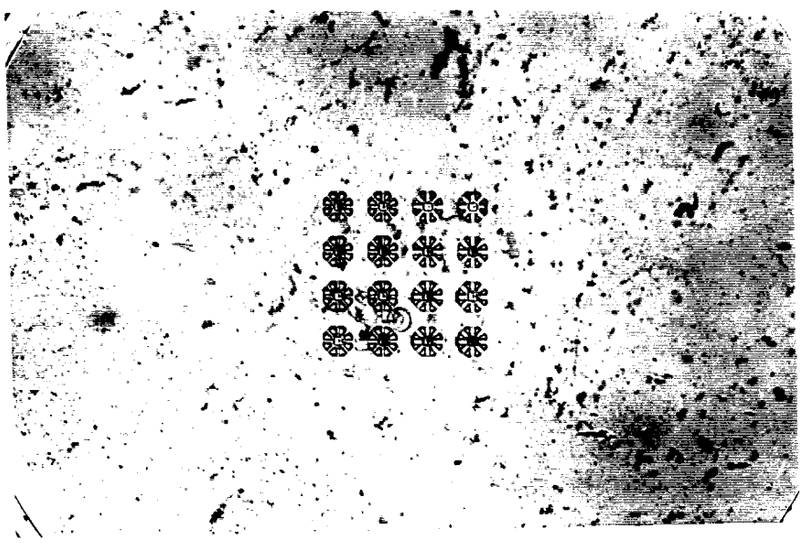
The pictures on the following pages show a complete mounted neurochip at decreasing levels of magnification. The first is a 270X picture of a fully platinized, canopy neurochip. The focus is at the top of the grillwork. The electrodes at the well bottoms are 15 μm down, so they appear out of focus. The central black spot is the platinized, 7.5 μm diameter active electrode area. The gray square marks the edge of the well where it contacts the canopy. There are wells with wide and narrow channels for ongoing long term studies of optimal channel width to minimize escape without inhibiting neural outgrowth and survival.

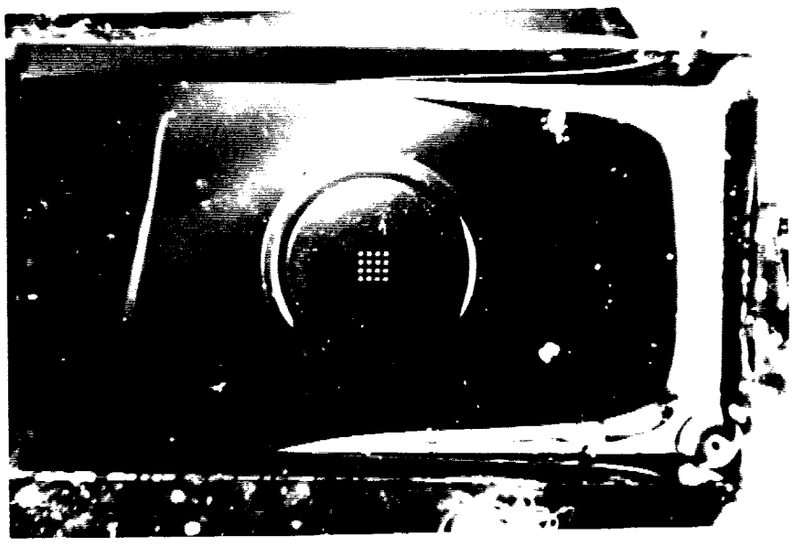
The second picture is at 67X and shows the basin area of the chip. The white arcs at the corners of the picture are the edges of a silicon nitride barrier 2 mm in diameter which serves to separate the cells in the wells from the rest of the feeder culture. After loading the wells, this 2mm diameter region is vacuumed free of extra cells, and a high density of cells is allowed to grow in the rest of basin.

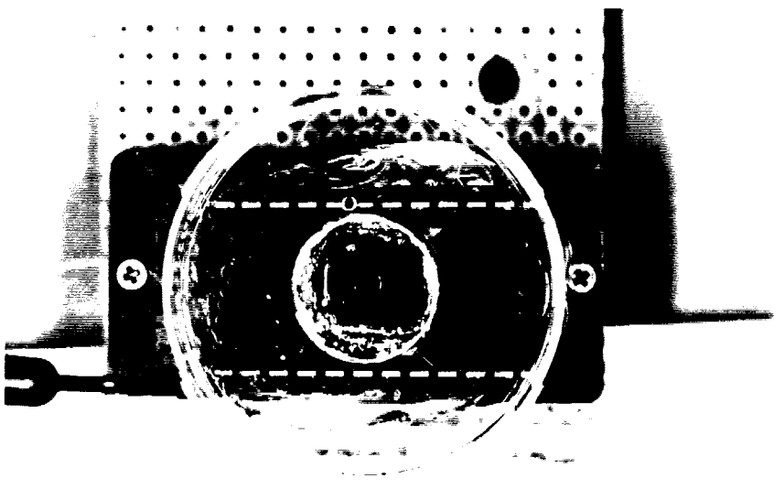
The third picture shows the entire basin at 13X. The 16 wells can be seen at the center, as well as the 2 mm diameter circle surrounding the wells. The central circle, upon which the cells in the wells grow, is bare silicon. The surrounding area is silicon nitride. The entire basin is 8 mm x 4 mm wide, and approximately 0.5 mm deep. The bright area at the right edge of the basin is a small amount of Silastic poting material which overflowed into the basin.

The fourth picture shows the entire chip assembly at 1.7x, plugged into a zero-insertion-force (ZIF) socket on a circuit board. A 35 mm petri dish with a half-inch diameter hole through the bottom is sealed to the chip with Silastic. The two rows of pins to which the wire bonds are attached are clearly visible on either side of the chip. The 1 mil gold lead wires don't show up well at this magnification. The ZIF socket makes a simple, reliable connection to the component carrier, and since it does not go into the incubator, it will not corrode.









After a long period of evolution, a neurochip mounting and lead attachment system has finally been developed which is durable, convenient, and stable for long term experiments.

Platinization

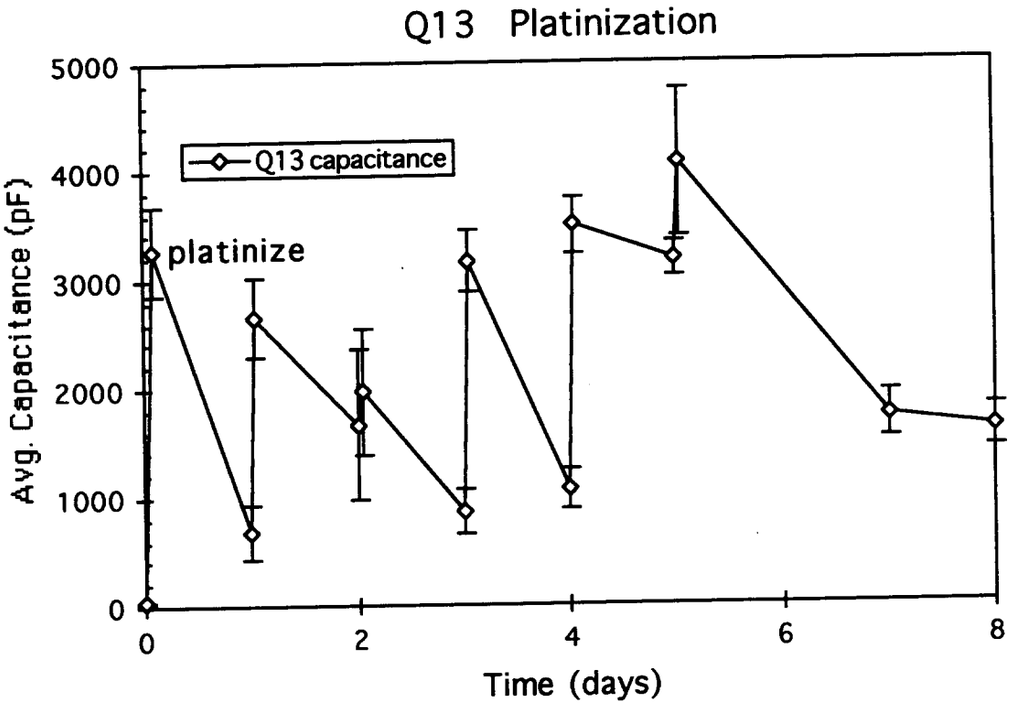
The active area of the electrodes in the new canopy-style chips is about 50 μm^2, for a theoretical capacitance of about 50 pF. Actual unplatinized capacitances are 15-30 pF. For recording, the electrode capacitance must exceed the shunt and pre-amp capacitances (approximately 150 pF) , to avoid significant signal loss. For stimulation, however, we estimate conservatively that a capacitance of 1000 pF would be desirable.

Historically, the capacitance of gold electrodes has been increased by a factor of 10-20 by platinization. In our system, we typically see an initial increase of a factor of 200 or more (to 3000-4000 pF), but this capacitance increase quickly degrades within a day or less to a factor of 10-20 (to 150-400 pF). To ensure enough capacitance for stimulation, we need to make the initial increase more stable, so that there is enough capacitance after 2-4 weeks in culture to stimulate neurons. We found that platinizing the electrodes daily for 5 days accomplishes this requirement. In the graph below, the neurochip electrodes were measured before and after the daily platinization over 5 days. The daily fall in capacitance is smaller over the course of this treatment, and surprisingly, the platinized capacitance continues to rise. At the end of the treatment, the capacitance falls to a stable level of 1500 pF.

The figure below illustrates the change in capacitance over an eight day period, with repeated platinization, for a typical electrode. The platinizing protocol was:

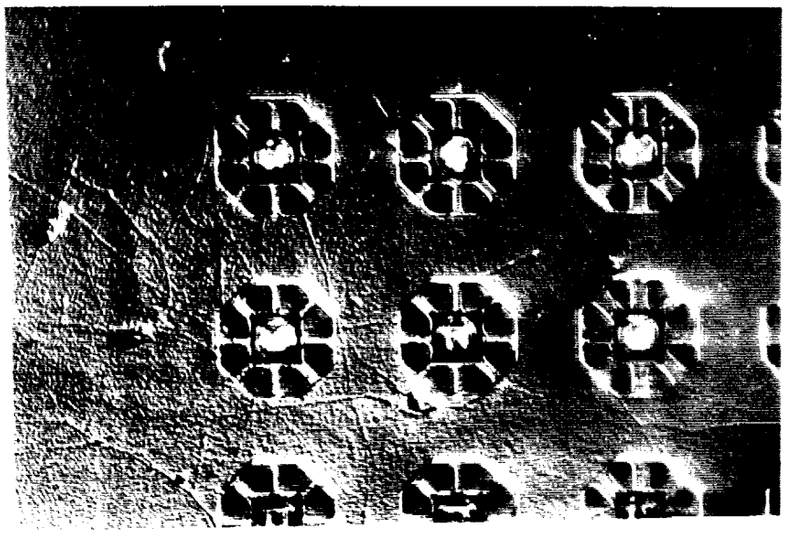
(1) 1% chloroplatinic acid, 0.0025% hydrochloric acid, 0.01% lead acetate in water.

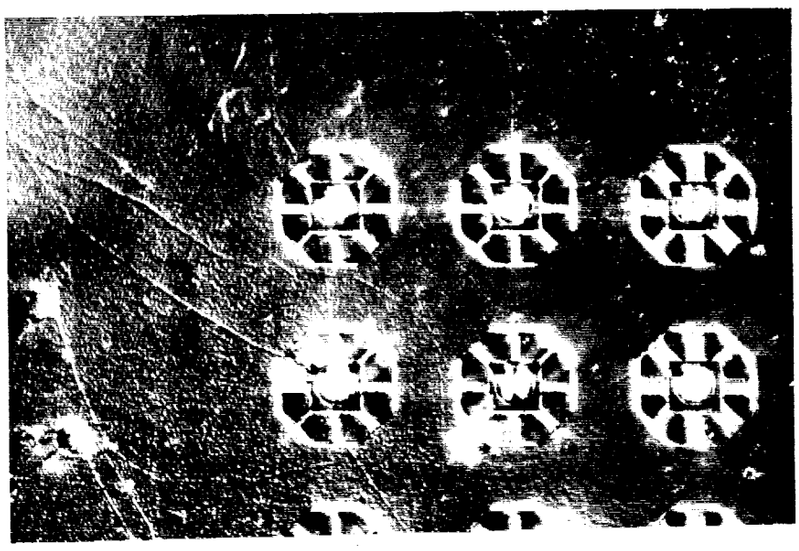
(2) 8V across a 100 MΩ cxurrent-limiting resistor for 10 sec. ($8x10^{-7}$ C)

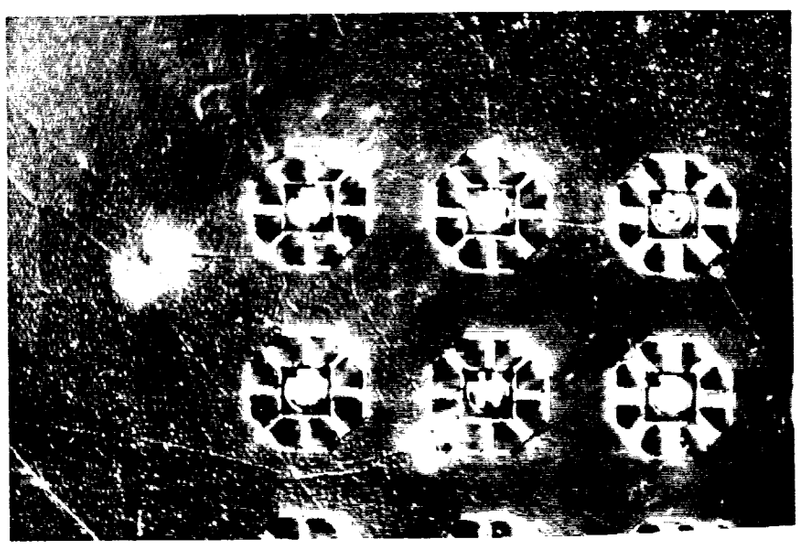


SCG's in Neurochips

In general, SCG's are easier to culture and to use for electrophysiology than are hippocampal neurons. Therefore, our plan has always been to use SCG's for the initial tests of the neurochip. Unfortunately, SCG's show a great propensity for escaping from the wells at a very early stage of development. The pictures on the following page show part of a neurochip with SCG's loaded into its wells. The first one is after 24 hours in culture (magnification 250X). In a reproduced picture, it may not be possible to detect all the features. The cell in row 2, column 2 has already escaped, while the cells in (1,1), (2,1), and (1,2) all contain growing cells. Several processes can be seen growing through the tunnels, but an approximately equal number emerge from the central holes. In particular, note that the cell in well (1,2) has at least 3 processes on top of the well.







At 48 hours in culture (second picture), the cell in well (1,2) is sitting on top of the grillwork. The diameter of the cell body is approximately 20 μm, so it was not forced out by its size. However, it is possible that the cell body was large enough to touch the bottom of the grillwork while it was sitting at the bottom of the well, and thereby grow processes out through the central hole. The third picture shows the situation at 3 days in culture. Processes on top of the wells greatly outnumber processes through the tunnels. By one week in culture, essentially all living cells have escaped the wells, and processes grow indiscriminantly over the grillwork of nearby wells.

Since some cells do remain in wells for the first week, it should be possible to stimulate them and record using the neurochip during this time. However, while SCG's appear to grow and develop quickly enough during the first week, their electrical activity takes weeks to develop. By the end of the first week, resting potentials range from -20 to -40 mV, and action potentials are small (30 to 40 mV spikes) and quite slow (rising phase 1-2 ms long). These factors conspire to reduce the current by nearly an order of magnitude, so that the extracellular signal would be indistinguishable from noise. Recording attempts using SCG's in wells known to have good electrodes failed, apparently for this reason. Therefore, we will attempt to use hippocampal neurons in neurochips during the next quarter.

Hippocampal cell culture for Neurochips

It is desirable to have cultures of hippocampal pyramdal cells which have few if any glia, and originally we used the system developed by Brewer in which a serum-free medium was used which was not compatible with glial cell proliferation. However, we have found that this medium does not support good long-term (greater than one week) growth and survival. The other known technique for growing "pure" hippocampal cultures was pioneered by Banker. It involves growth of cells in serum free medium in close proximity to previously prepared confluent glial cultures. The glia apparently provide unknown diffusable molecules which are needed for long-term growth and survival.

In Banker's lab, the hippocampal neurons are grown on cover slips which are inverted at a distance of about 1 mm over a bed of glia. For neurochips, we have developed a more appropriate system, with glia on cover slips supported over the neuron culture. The glia are on 22 mm square slips which nicely fit into the neurochip culture dish, held away from the silicon by 1mm diameter plastic sphers at the corners. Very good growth and survival have been achieved, with Banker's serum-free medium, and perhaps even better with Brewer's medium, so long as the glia are present. In order to view the neurochip culture, and to manipulate electrodes over it, the glial cover slip needs to be removed temporarily under sterile conditions.

Fabrication

During this quarter, several runs of dummy neuroprobes and neurochips have been completed. Yields for both devices have been significantly improved. We are now confident that the present canopy fabrication process can produce high quality devices with high yields.

Supplementing the dummy probes produced last quarter, several more of these wafers have been brought to the end of the fabrication process. Prior to the final step (i.e. RIE definition of the probe shape) a survey of the quality of the wells and the likelihood of successful freeing of the probes from two wafers was conducted. The survey result is shown in the table below. Considering this is the first run of dummy probes using the abbreviated version of the fabrication process that will be used to produce real probes, the yields are quite good. The final RIE step did damage some of the probes but that number is relatively small.

Probe Quality	Total Number	Yield
15 to 16 Good Wells	202	52 %
> 10 Good wells	81	21 %
> 5 Good Wells	75	19 %
< 5 Good Wells	34	9 %

After placing an order last November, our wafer stock has finally been replenished with twenty new wafers. The timing of their arrival was nearly perfect as the last epi wafer from our wafer bank was used just a few weeks earlier. These wafers have a 4μm B-Ge etch stop buried beneath 16 μm of lightly doped silicon. We have moved from a B-only to a B-Ge etch stop for two reasons. First, membranes formed from B-only wafers display a "linen" pattern

when viewed with a microscope. This pattern makes observation of neural processes extremely difficult. Second, we have found that B-Ge is a much better EDP etch stop than is B-only. B-Ge silicon last many hours in EDP as compared to the one to two hours typical for B-only silicon.

With the new wafers, a run of real neurochips was started. This run, only the second using the real canopy chip fabrication process, has shown the present process to be well developed. The fabrication complications experienced during the first run have all been resolved allowing all of the wafers to be brought to the end of the process with near perfect features and the potential for better than 85% yield. As fate would have it, however, an unexplained phenomenon encountered in the last step of the process has lessened the yield considerably.

In the last step the neurowells are to be formed in the membrane using an anisotropic EDP silicon etch. This etch is not working as it should. A previous step in the process rules out that that the problem is due to the new batch of epi-wafers. This leaves us with the possibility of a processing error, of an incompletely removed deposited layer, or an unknown surface contaminant or of a problem with the EDP. All of these possible causes have been examined with varying results. Of the three wafers in the run, only one has had wells successfully created. The remaining two, although they have received the same treatment as the one completed wafer, stubbornly refuse to be etched by EDP or for that matter, any silicon etchant. We are still attempting to bring the two wafers to completion.

From the one wafer that has been successfully completed, a fair number of neurochips have been produced. Twelve are near perfect with all sixteen wells fully formed and all electrodes intact and centered at the bottom of the wells. Additionally, there are twelve with only slight defects, such as an unformed well or speckled field silicon are available. While not perfect, these are more than satisfactory for initial electrophysiological experiments. Finally, eleven of the neurochips from the wafer are unusable. It should be noted that most of these were damaged by the relatively rough treatment they received while tests were conducted to find a way to get the wells to etch. The table

below summarizes the results from this wafer. The yield is calculated based on a maximum theoretical chip yield of 35.

Chip Quality	Total Number	Yield
Near Perfect	12	34 %
Good	12	34 %
Poor	11	31 %

Presently, work to determine what is preventing the wells from being etched is being conducted.

In Vivo Studies

During the last quarter we examined the long-term survival of septal neurons in dummy probes placed into the hippocampus.

Procedure for the examination of process outgrowth

As discussed in our previous progress report, fluorescence labeling of implanted neuron in both probes and graft aggregates has the potential problem that the degenerating neurons release the dye, which in turn may be incorporated by host cells. In addition, the fluorescent dye may decrease the viability of the grafted cells. During the last period, therefore, we have concentrated our efforts to examine the fate of unstained septal cells implanted *in vivo*.

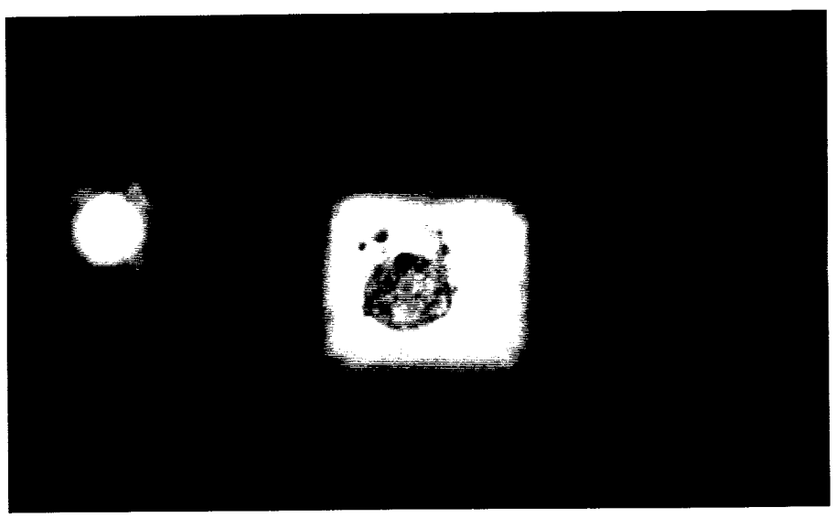
The preparation of septal cell suspensions and the loading cells into wells were the same as in previous experiments. In these experiments, the first version of Caltech probes with large and smaller holes were used. Several unstained septal neurons were placed in the large holes and typically a single cell into the small holes. The neurons were cultured 24-48 hours in the incubator after which the probes were implanted. The adult recipient rat was deeply anesthetized, the scalp was opened and the brain meninges were removed by scissors. The fimbria-fornix was removed by suction. This procedure assured complete denervation of the dorsal hippocampus from its subcortical inputs, including the medial septum. The goal of this procedure was to remove all cholinergic fibers from the hippocampus and allow assessment of axonal outgrowth from the grafted cholinergic cells. The surface of the brain was covered by saline. The probes with the neurons were placed into the brain with a stereotaxic holder in less than 5 minutes. The handle area of the probe was fixed to the skull with dental acrylic. The rats were allowed to survive for 4 and 8 months (2 rats in each group) after implantation.

The brains were cut by a Vibratome, using 100 μm sections and were stained with the modified Koella acetylcholinesterase method, as reported in previous progress reports. We were able to cut slices in several hemispheres in

such a way that the probe or very large part of it was contained in a single 100 μm section.

Altogether 7 probes in 7 hemispheres were examined. Although we have seen acetylcholinesterase fibers in 2 rats in the vicinity of the probe, their origin from the probe could not be convincingly demonstrated, since we could not identify a single major axon collateral emanating from a well. The failure of process outgrowth from the wells might be explained by assuming that the grafting neurons did not survive the transplantation procedure or died sometime after the grafting, as was discussed in several of our previous progress reports. Therefore, we carefully investigated the presence or absence of surviving neurons in the wells. By adjusting the focal depth and the intensity of transillumination, it was possible to see through the bigger size holes of the probe and to some extent visualize the content of smaller holes.

None of the small holes, into which only a single neuron was placed during the culturing phase, contained cells, or their presence could not be verified. In contrast, in one third of the larger size holes the presence of one to several cells could be unequivocally demonstrated. The figure below shows a stained cell body transilluminated through the well bottom. Immersion oil-examination (100X) of these neurons failed to reveal processes leaving the wells. In some cases, small and short processes were clearly visible but in the majority of cases the neurons had a round shape.



These findings have important implications for the development of a successful cultured neuron probe. First and most unexpected is the observation that neurons can survive several months without growing processes. Second, the size of the wells may be critical and perhaps more than a single neuron per well is required for survival. Single neurons did not survive. Third, the cells were implanted into holes of the first generation holes. As reported earlier, we had difficulty of culturing neurons in these probes and identified the cause: a minute but significant overhang at the edges prevented process outgrowth from the wells. Whereas subsequent versions eliminated this problem, we never tried to implant more than a single neuron per well using the new probes. An obvious conclusion for future experimentation is that we should try to implant several cell into wells and increase the hole size if necessary.